Pak. J. Biotechnol. Vol 15 (2) 553-560 (2018) *ISSN Print: 1812-1837*
www.pjbt.org *ISSN Online: 2312-7791*

CORRELATION BETWEEN BIOFILM FORMATION AND BACTERIOCIN PRODUCTION BY *LACTOBACILLUS ACIDOPHILUS*

Jnan Jafr Baksh and Amal Aziz Kareem

[1]Department medical analysis, Health and medical technology, Baghdad, Iraq.
E. mail: jnanjfralmndlawy252@gmail.com

Article received 20.4.208, Revised 2.6.2018, Accepted 12.6.2018

ABSTRACT

The present study was carried out to investigate the Correlation between biofilm formation and bacteriocin production by Lactobacillus acidophilus A total of 214 vaginal swab of healthy women without vaginitis and/or urinary tract infection were collected from hospitals in Baghdad city .Vaginal swabs of each women inoculated in MRS broth medium, after 24 h incubation in the presence of 5% CO_2 the specimens were sub-cultured on MRS agar and 49 samples from 104 samples were *Lactobacillus acidophilus*, while negative growth were 110 samples. The first identification presented then using conventional polymerase chain reaction (PCR) with specific primers gene which showed that 11 isolates were *Lactobacillus acidophilus* carried bacteriocin gene also 11 *L. acidophilus* isolates characterized by their ability to inhibit the growth of *Staphylococcus aurous , Pseudomonas aeroginosa , Escherichia coli* through the production of bacteriocin and all *Lactobacillus acidophilus* isolates form biofilm at different levels (weak, moderate and strong) were produced bacteriocin. This study suggested highly significant difference and strong correlation (r= 0.914; P = 0.00; P<0.01) between biofilm formation and bacteriocin production.

Key words: *Lactobacillus acidophilus*, biofilm, bacteriocin, vagina

INTRODUCTION

Human microbiota is a collective of microorganisms that live in human host and most of these microbes associated with human kind influence in maintaining processes essential for a healthy body and colonize the conjunctiva, oral cavity, gastrointestinal tract, skin and vagina (Selle and Klaenhammer, 2013).

Lactobacillus species is dominated in vaginal healthy females of reproductive age, the vaginal flora in healthy women were characterized by different approaches and had been found that Lactobacillus, acidophilus is the major dominated (Kurakawa et al., 2015). Lactobacillus acidophilus is useful supplies because it could maintenance colonization and longer strength in the mucosa of the host which prevent colonization by bacterial pathogens (Terraf et al., 2012) and inhibition activity against common human pathogens through their ability to produce antibacterial substances for example bacteriocins therefore used as probiotic (Linsalata et al., 2010). Lactobacillus acidophilus formed the biofilms on biotic surfaces (polystyrene or glass (Fernández et al., 2015).

The extracellular polysaccharide (EPS) produced by biofilm forming strain is able to inhibit the biofilms formation by certain pathogens (Ramos et al., 2012) and has ability to bacteriocins production in addition to their non-toxic property on eukaryotic cells and the greatly broader inhibitory spectra make bacteriocins (Balciunas, 2013).

Bacteriocins are proteinaceous antibacterial compounds that show bactericidal activity against species closely related to the producer strain (Signoretto et al., 2000). Finally, the genetic determinant for bacteriocin production can be either plasmid or chromosomally encoded (Klaenhammer,1993). Lactobacillus acidophilus produces plasmid associated bacteriocin, in addition to the gene may be part of transposons (Dufour et al., 2000).

MATERIALS AND METHODS

Isolation and identification: Vaginal specimens were obtained, from 214 women between the ages of 18-50 years with healthy vaginal environments without vaginitis and /or urinary-tract infection (UTI). The samples were diluted to MRS broth, then incubated at 37◦C for 24 h under anaerobic condition in the presence of 5% CO2 (Sneath et al., 2009).

The identification of the genus Lactobacillus done by PCR using specific primers

F:5-TGCAAAGTGGTAGCGTAAGC-3

R: 5-CCTTTCCCTCACGGTACTG -3.

Brolazo et al., (2011) prepared depending on the manufacturer's instructions (Alpha DNA /Canada) and using DNA Ladder 1Kb.

Table1: PCR amplification program

Steps	Temperature	Time	Number of cycles
Initial Denaturation	94 ºC	3min	One cycle
Denaturation	94 ºC	30sec	35cycles
Annealing	57 ºC	60sec	
Extension	72 ºC	30sec	
Finally Extension	72 ºC	5min	One cycles

PCR product was analyzed by gel electrophoresis in 2% agarose containing red safe TM (Nucleic acid staining solution) (Branco *et al.*,2010).

2.2 Detection of bacteriocin gene by (PCR): Using specific primers (Ventura *et al.,* 2001)

F:5- AAGAGTTTG ATCCT GGCTCAG -3

R:5-CTACGGCTACCTTGTTACGA-3

Primers were obtained from Promega, USA for detection of bacteriocin gene of *Lactobacillus acidophilus.*

Table 2: PCR amplification progra

Steps	Temperature	Time	Number of cycles
Initial Denaturation	95ºC	3min	One cycle
Denaturation	95ºC	30sec	35cycles
Annealing	61ºC	40sec	
Extension	72ºC	1min	
Finally Extension	72ºC	5min	One cycles

The amplified PCR products were checked for the expected size on 1% (w/v) agarose gel and visualized after staining with Red Safe under ultraviolet transillumnater (Sambrook and Rusell, 2001).

Biofilm formation assay is done by pure cultures of bacteria and quantified in polystyrene microtiter plates after adjusted by MacFerland tube (0.5 concentration) (Stepanovic, 2004).

2.4. Effect of some factors on biofilm formation:

I) pH: MRS broth medium was adjusted to different pH values 2, 4, 6, 7 and 8. This was distributed in sterile test tube then over- night culture of bacteria diluted and adjusted by McFarland tube to be 0.5 concentration, then 20µL from diluted and 180µL from MRS broth to obtain dilution 20/ 00µL was transferred to each well in a 96 wells dish, the microtiter plate was incubated at 37ºC for 24 hrs.

II) Temperatures: As the same method above just adjusted temperature at 34, 37 and 39ºC

III) Incubated at different periods of time: As the same method above just adjusted incubation periods 18, 24 and 48 h at 37ºC.

IV) Carbohydrate concentration (1%glucose): MRS broth tube was added 0.5 ml of 1% glucose sterilized through passing during milipore filter 0.24 mm in diameter then inoculated with each isolate and incubated at 18hrs.

Bacteriocin production

The bacterial were grown in MRS broth for 18h at 30°C. The cultures were centrifuged at 6000rpm/15 min/4°C) to obtain a cell free supernatant and the supernatants were filter-sterilized by passing through a sterile 0.2 mm pore size filter then pH of the supernatants was adjusted to 6.5 with 10 N of NaOH (Dunne *et al.*, 2001), supernatants against several indicator bacterial spp. was then performed (*Escherechia coli*, *Pseudomonas aerogenosa* and *Staphylococcus aureas).* Agar-well diffusion assay was used by aliquots of 50µl of the sterile supernatant were placed in 5 mm diameter wells on Muller-Hinton agar plates. The previously seeded with the respective indicator bacteria. After incubation18h at 37°C, the diameters of the zones of growth inhibition were measured (Ogunbanwo *et al.*, 2003).

Effect of different factors on bacteriocin production

(I) pH: MRS broth to special pH levels of (4, 6,7, and 8), each tube was inoculated with bacterial growth and incubated at 37°C for 18h.

(II) Temperatures: MRS broth 5ml was inoculated with each isolate and incubated at diverse temperatures such as 34°C, 37°C, and 39°C for 18h to study the effect of different temperatures on the bacteriocin production.

(III) Incubation time: MRS broth tube was inoculated with each isolate and incubated at diverse incubation periods such as 18, 24, and 48 hrs.

(IV) Carbohydrate concentration (1%glucose): MRS broth tube was added 0.5 ml of 1% glucose sterilized through passing during milipore filter 0.24 mm in diameter then inoculated with each isolate and incubated at 18hrs (Kandler and Weiss, 1986; McFadden, 2000).

Statistical Analysis: The usual statistical methods were adjusted in order to assess and analyze the results according to (Ying, 2015).

RESULTS

Table 3: Mean age / Year comparison among growth of genus *Lactobacillus* results

Growth of *Lactobacillus*	**No. of isolates**	**Percentage**	**Kolmogorov-Smirnov Test (P-value)**
***Lactobacillus* species**	**55**	**25.7%**	**P=0.00 Highly sign. (P<0.01)**
L. acidophilus	**49**	**22.9%**	
No growth	**110**	**51.4%**	
Total	**214**	**100%**	

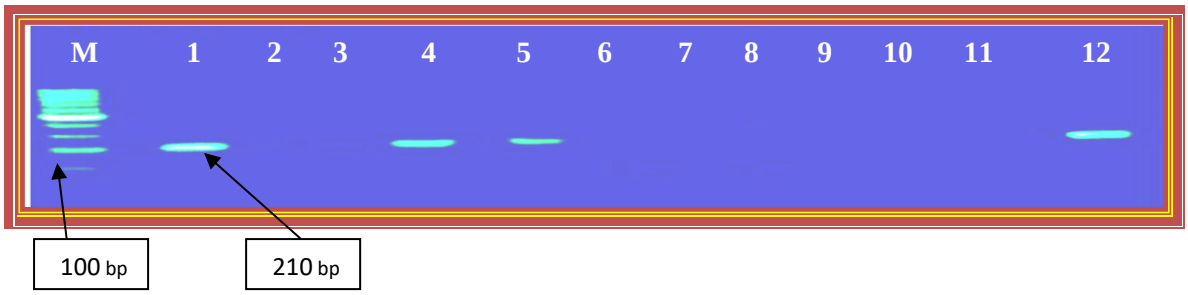

Figure 1: *Lactobacillus acidophilus* identification bands at 210bp (From left to right 1, 4, 5, 12 while no present band at 2, 3, 6, 7, 8, 9, 10, 11) by using ladder 1kb and 2% agarose for electrophoresis.

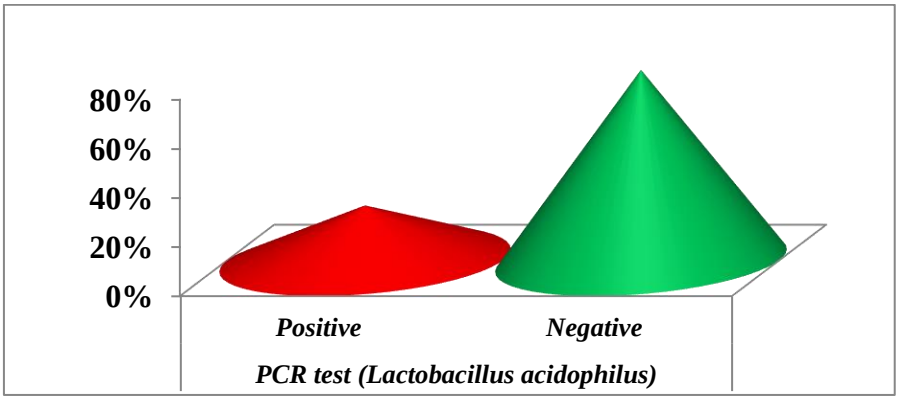

Figure 2: *Distributions of Lactobacillus acidophilus isolation according to PCR test results.*

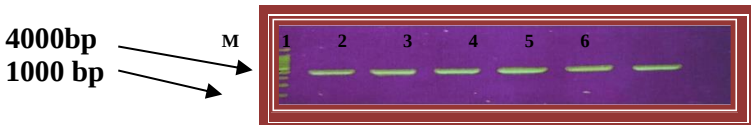

Figure 3: *Lactobacillus acidophilus* bacteriocin gene band 4000 bp at lines (1-6) by using ladder 10 kb and 1% agarose for electrophoresis.

Table 4: Distributions of classes of *Lactobacillus acidophilus* biofilm formation.

Factors		**Classes of *L. acidophilus* biofilm formation**		
		Weak biofilm OD630 (< 0.1)	Moderate biofilm OD 630 (0.1 - 0.5)	Strong biofilm OD630 (> 0.5)
Biofilm of *Lactobacillus acidophilus*	N	2	3	6
	%	18.2%	27.3%	54.5%

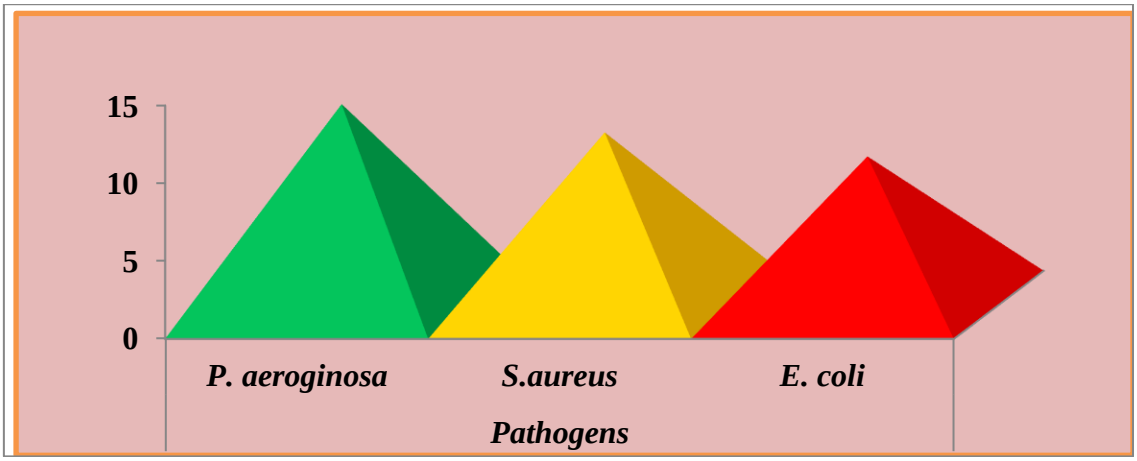

Figure 5: Determination *L. acidophilus* bacteriocin inhibition effect against some pathogens (1: *Pseudomonas aeroginosa ,2: Staphylococcus aureus,* 3*: Escherichia coli)* by agar diffusion assay.

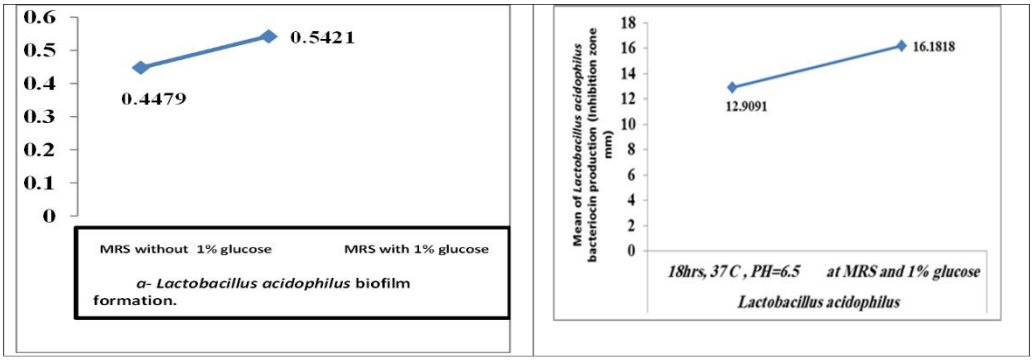

Figure 4: MRS with1% glucose effects on *Lactobacillus acidophilus* bacteriocin production Inhibition zone mm. (a: Biofilm formation b: Bacteriocin production).

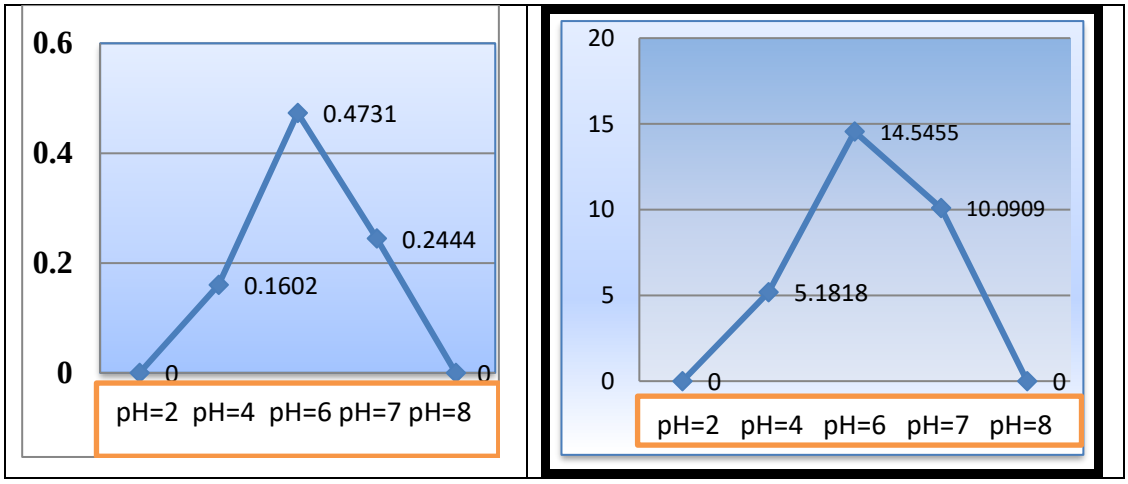

Figure 6: pH effects on *Lactobacillus acidophilus* from left to right a. Biofilm formation and b. Bacteriocin production (Inhibition zone mm).

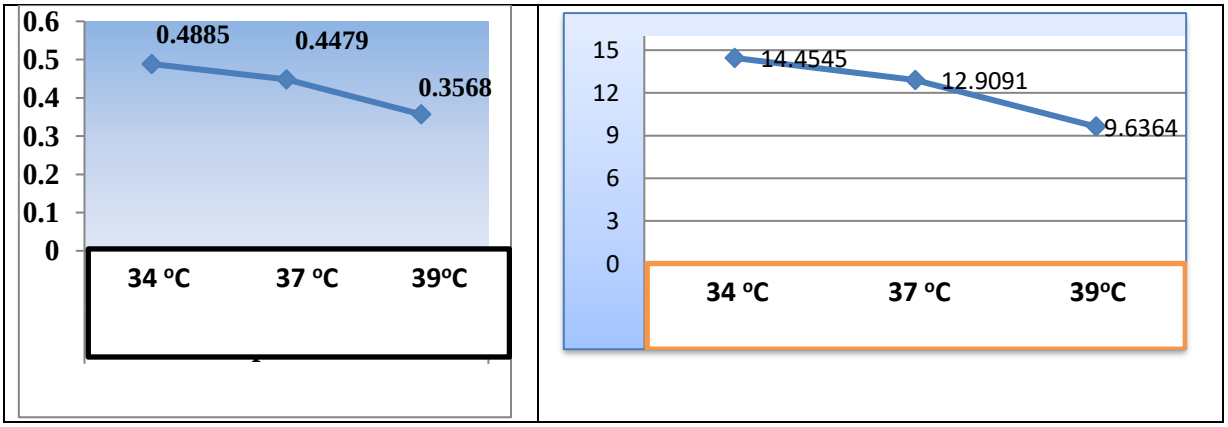

Figure 7: Temperatures effects on *Lactobacillus acidophilus* a. Biofilm formation and b. Bacteriocin production (Inhibition zone mm).

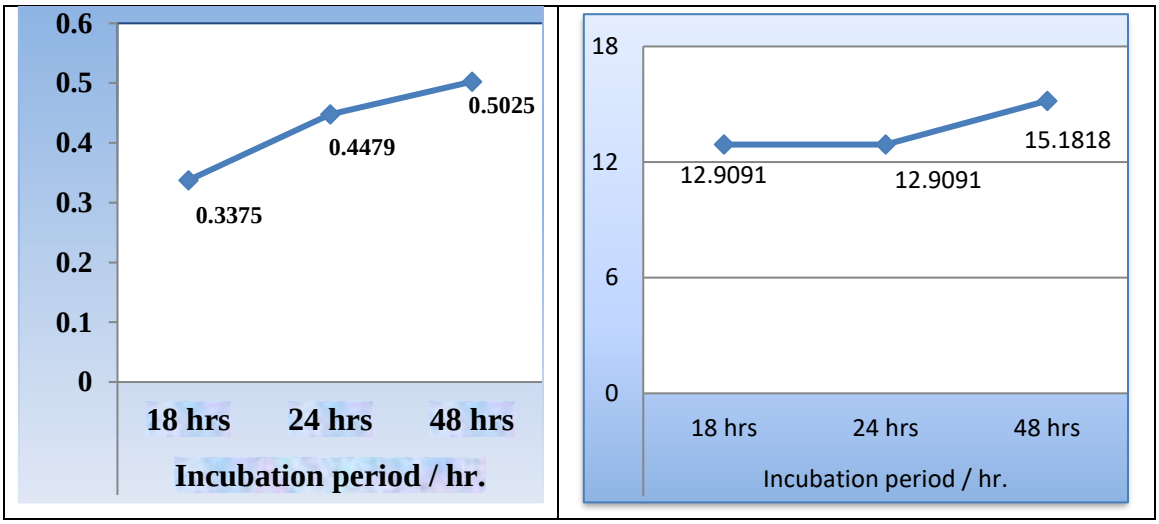

Figure 8: Incubation period / hour effects on *Lactobacillus acidophilus* from left to right a. biofilm formation and b. bacteriocin production (Inhibition zone mm).

Table 5: Clusters and sources of isolates with their description depending on biofilm formation and bacteriocin production at the same experiment conditions.

Cluster	No. of isolates	Source of isolate	Description
C1	**J1, J2, J7, J8, J9, J 11**	**Healthy young women ages between 18-22 year**	**All isolates strong biofilm formed and more bacteriocin production**
C2	**J4, J5, J10**	**Healthy young women 26-38 year with menstruation**	**All isolates s moderate biofilm formed and bacteriocin produced.**
C3	**J6** **J3**	**Healthy woman with hormonal contraceptive intake 27 year** **Healthy old women 43 year.**	**Isolates weak biofilm formed and less bacteriocin production**

J: Jnan

Table 6: Correlation between biofilm formation and bacteriocin production by *Lactobacillus acidophilus.*

Pearson Correlation		Biofilm at 48hrs, pH=6,34ºC MRS and 1% glucose
Inhibition zone at 48hrs, pH=6, 34 º C MRS and 1%glucose	r	0.914
	P-value	P=0.00 Highly sign. (P<0.01)

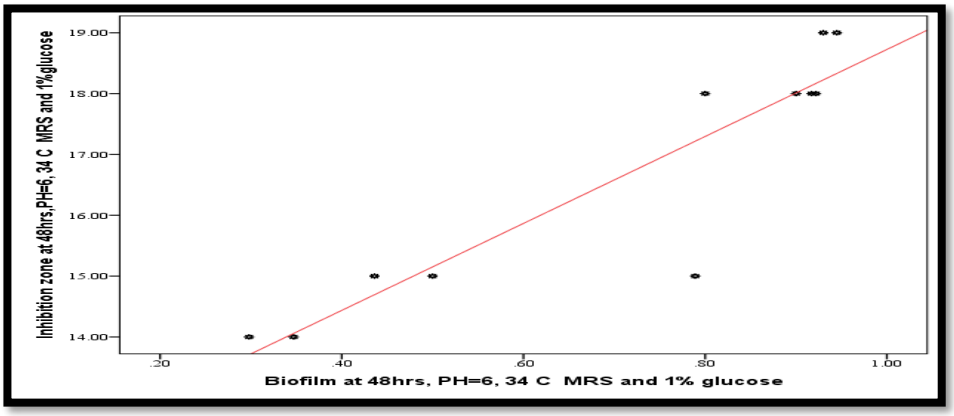

Figure 9: Correlation between biofilm formation and bacteriocin production by *Lactobacillus acidophilus.*

DISCUSSION

Depending on the first identification of bacteria, all 49 *Lactobacillus acidophilus* isolates were amplified with specific primers; 11 isolates 22.4% produced a product 210 bp as in figure1. The vaginal environment undergoes main compo-

sitional changes during a woman's life from childhood until puberty, the limited presence of estrogens suggests a low vaginal bacterial content, which occurs during reproductive years or through menopause (Jaisamrarn *et al.,* 2013). It is also that several factors (Menstrual cycle, coition, use of antibiotics, use of intra vaginal products for doughing (Javier *et al*., 2014) and breastfeeding (Boskey *et al.,* 1999) influence the balance of the vaginal microbiota.

Presented the results all 11 *Lactobacillus acidophilus* isolates were molecular identified carrying bacteriocin gene by amplified with the bacteriocin gene primers, all 11 isolates produced a product 4000bp as in (figure 2). Lactic acid bacteria (LAB) are produced some substances such as organic acids hydrogen peroxide, carbon dioxide and bacteriocins (Dunne *et al.,* 2001).

Bacteriocins have been reported to be inhibitory against several other bacteria, most of bacteriocins produced by Gram positive bacteria are from lactic acid bacteria (Graneau *et al.,* 2002).

Some LAB bacteriocins can inhibit the growth of Gram-positive pathogenic bacteria and also inhibit the growth of some Gram-negative species, therefore, such these lactic acid bacteria can be used as probiotic (Topisirovic *et al.,* 2006).

The isolates shown a different capacity to bacteriocin production under the same conditions of experimentation, the results represented the cell-free-supernatants exerted varying inhibitory effect on the indicator pathogens and inhibition was assessed against *Escherechia coli, Pseudomonas aerogenosa* and *Staphylococcus aureas* and this study agree with Abo-Amer (2007) and Kyoung-Sik *et al.,* (2007) Abo-Amer (2007) and Kyoung *et al.,* 2007).

Table 6 represented positive strong correlation between *Lactobacillus acidophilus* biofilm formation and bacteriocin production. The study included effect different conditions on *Lactobacillus acidophilus b*iofilm formation as following:

Figure 9 presented effect using MRS broth medium with 1% glucose on biofilm formation was higher than MRS medium without 1% glucose.

In this study represented a microtiter plate format assay was used to assay biofilm formation on MRS medium, the isolates showed increase in nutrient concentration increased biofilm formation this is accepted with study by Rochex and Lebeault (2007).

Figure 6 represented the ability of *Lactobacillus acidophilus* for bacteriocin production to different concentration of pH 2, 4, 6, 7 and 8. The maximum level in pH=6 was larger than pH =4 and pH=, while no bacteriocin production in pH=2 and pH=8.

Figure 7 revealed that the bacteriocin production at 34ºC was higher than bacteriocin production at 37ºC and 39ºC, change in human body temperatures due to different causes environment factors such as (diet or fasting) or aging or pathology or disease (Gregory Kelly, Vice, 2006) when pathogens enter human body occur changes in temperature begin human body formation of natural protection factors (Bacteriocins production) inhibit the growth of the human pathogenic bacteria for examples *Escherechia coli, Pseudomonous aeruginosa* (Forestier *et al.,* 2001).

Figure 8 represented effect incubation period/hour on *Lactobacillus acidophilus* bacteriocin production was at 48hrs larger than 18 and 24hrs. The Incubation periods used in this study were monitored in limited time because it is not suitable to increase the time since the wells contain limited nutrients for bacterial growth because lack of nutrients may stimulate the bacteria to detach from the surface (Hunt *et al.,* 2004; Sawyer and Hermanowicz, 1998).

Table 7 revealed that there were a statistically highly significant difference & strong positive (a proportionate) correlation (r= 0.914; P=0.00; P< 0.01) between biofilm at 48hrs, pH=6, 34ºC MRS and 1% glucose and iinhibition zone at 48hrs, pH = 6, 34ºC MRS and 1%glucose.

Conclusion: There is a correlation between biofilm formation and bacteriocin production of *Lactobacillus acidophilus* at 48hrs, pH=6, 34ºC MRS with 1% glucose and inhibition zone at 48hrs, pH=6, 34ºC MRS with 1% glucose.

REFERENCES

Abo-Amer, A.E., Molecular characterization of antimicrobial compound *acidophilus* GP1B. J. Microbiol. Biotechnol. 17: 774-783 (2007).

Balciunas E.M., Martinez F.A.C., Todorov S.D., de Melo Franco B.D.G., Converti A. and R.P. de Souza Oliveira, Novel biotechnological applications of bacteriocins. Food Control. 32:134-142 (2013).

Boskey, E.R., Telsch, K.M., Whaley, K.J., Moench, T.R. and R.A. Cone, Acid production by vaginal flora in-vitro Is consistent with the rate and extent of vaginal acidification. Infection and Immunity 67: 5170-5175 (1999)

Branco, K., Nardi, R., Moreira, A., Farias, L., Nicoli, J. and M. Cavaih, Identification and in vitro production of *Lactobacillus* antagonists from women with or without bacterial vaginosis. Braz. J. Med. Biol Res. 43: 100 - 879 (2010).

Brolazo, E., Leite, D., Tiba, M., Villarroel, M., Marconi, C. and J. Atonio, Correlation between Api 50 CH multiplex polymerase for identification of vaginal lactobacilli in isolates. Braz. J. Microbial. 42: 225-232 (2011).

Dufour, A., Rince, A., Uguen, P., and J.P. Le Pennec, IS1675 a novel lactococcal insertion element, forms a transposon like structure include in the lacticin 481 lantibiotic operon. J. Bacteriol. 82: 5600-5605 (2000).

Dunne,C., OMahony, L., Murphy, L. and G. Thomton et al., In vitro selection criteria for probiotic bacteria of human origin :correlation with in vivo findings. Am. J. Clin. Nutr. 73: 386-392 (2001).

Fernández, M., Smid, E., Abee, T. and M. Nierop, Characterization of biofilms formed by *Lactobacillus plantarum* WCFS1 and food spoilage isolates. Int. J. Food Microbiol. 207: 23–29 (2015).

Forestier, C., Champs C.D., Vatoux C. and B. Joly, Probiotic activities of *Lactobacillus casei rhamnosus*: in vitro adherence to intestinal cells and antimicrobial properties. Research in Microbiology.152:167–173 (2001).

Graneau S, Martin NI and Vederas JC. Two peptide bacteriocins produced by lactic acid bacteria. Biochimie 84: 577-592 (2002).

Gregory Kelly, N.D.V., Body temperature variability (Part1): Areview of the history of body temperature andits variability due to site selection, Biological thythms, fitness, and aging. Alternative Medicine Review 5(11): 278- 292 (2006).

Hunt S.M., Werner E.M., Huang B., Hamilton M.A. and P.S. Stewart, Hypothesis for the role of nutrient starvation in biofilm detachment. Appl. Environ. Microbiol. 70: 7418-7425 (2004).

Jaisamrarn, U., Triratanachat, S., Chaikittisilpa, S., Grob, P., Prasauskas, V. and V. Taechakraichana, Ultra- Low-Dose Estriol and lactobacilli in the local treatment of postmenopausal vaginal atrophy. Climacteric 16: 34-355 (2013).

Javier Haya, J., García, A., López-Manzanara, C., Balawi, M. and L. Haya, Importance of lactic acid in maintaining vaginal health. A review of vaginitis and vaginosis etiopathogenic bases and a proposal for a new treatment. Open Journal of Obstetrics and Gynecology 4:787-799 (2014).

Kandler O., Weiss N., Sneath P.H.A., Mair N.S., Sharpe M.E. and J.G. Holt (eds). Regular, non-sporing, gram-positive rods. Bergey's Manual of Systematic Bacteriology. Williams and Wilkins MD. USA.Vol. 2 (1996)

Kurakawa, T., Ogata, K., Tsuji, H., Kado, Y., Takahashi, T., Kida, Y., Ito, M., Okada, N. and K. Nomoto, Establishment of a sensitive system for analysis of human vaginal microbiota on the basis of rRNA-targeted reverse transcription- quantitative PCR. Journal of Microbiological Methods 111: 39-104 (2015).

Kyoung-sick H., Kim Y., Kim S.H. and S. Oh, Characterization and Purification of Acidocin 1B, a Bacteriocin Produced by Lactobacillus acidophilus GP1B. J. Microbiol. Biotechnol. 17: 774-783 (2007).

Klaenhammer T.R., Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiological Review 12: 39-85 (1993).

Linsalata, M., Cavallini, A., Messa, C., Orlando, A. and F. Russo, Current Pharmaceutical Design 16: 847-853 (2010).

McFadden J.F., Biochemical tests for identification of medical bacteria (3ed), Lippincott Williams and wilkins, USA Microbiol. Rev. 2: 15-38 (2000).

Ogunbanwo, S.T., Sanni, A.I. and A.A. Onilude, Characterization of bacteriocin produced by *Lactobacillus plantarum* F1 and *Lactobacillus brevis* OG1. Afr. J. Biotechnol. 2(8): 219-227 (2003).

Ramos, A., Sesto Cabral, M., Noseda, D., Bosch, A., Yantorno and O., Valdez, Anti-pathogenic properties of *Lactobacillus plantarum* on *Pseudomonas aeruginosa*. The potential use of its supernatants in the treatment of infected chronic wounds. Wound Repair Regen 20: 552–562 (2012).

Rochex, A. and J.M. Lebeault, Effects of nutrients on biofilms formation and detachment of Pseudomonas putida strain isolated from a paper machine. Water Res. 41: 2885-2892 (2007).

Sambrook J. and D.W. Rusell, Molecular cloning: A Laboratory Manual, 3rd ed. cold spring harbor: cold spring harbor laboratory press, NY (2001).

Sawyer L.K. and S.W. Hermanowicz, Detachment of biofilm bacteria due to variations in nutrient supply. Water Sci. Technol. 37: 211-214 (1998).

Selle K. and T. Klaenhammer, Genomic and phenotypic evidence for probiotic influences of *Lactobacillus gasseri* on human health. FEMS Microbiol Rev 37: 915–935 (2013).

Signoretto, C., Lieo, M.M., Tafi, M.C. and P. Canepari, cell wall chemical composition of Enterococcus *faecallus* in the viable but non-

culturable state. Appl Environ Microbiol. 66 (6): 1953-1959 (2000).

Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and J.G. Holt, Bergeys manual of systematic bacteriology. Baltimore, Williams & wilkirs (2009).

Stepanovic, S., Cirkovic, I., Ranin, L. and M. Svabic-Vlahovic, Biofilm formation by Salmonella spp. and *Listeria monocytogenes* on plastic surface. Lett. Appl. Microbiol. 38:428-43 (2004).

Terraf, M.C., Juarez, M.S., Nader-Macias, M.E. and C. Silva, Screening of biofilm formation by beneficial vaginal lactobacilli and influence of culture media components. J. Appl. Microbiol. 113(6): 1517-1729 (2012).

Topisirovic, L., Kojic, M., Fira, L., Golic, N., Strahinic, I. and P. Lozo, Potential of lactic acid bacteria isolated from specific natural niches in food production and preservation. International Journal of Food Microbiology 112: 230-235 (2006).

Ventura M., Elli M., Reniero R. and R. Zink, Molecular microbial analysis of Bifidobacterium isolates from different environments by the species specific amplified ribosomal DNA restriction analysis (ARDRA). FEMS Microbiol. Ecol. 36:113-121 (2001).

Ying lu, Jiqian Fang, lu Tian and Huajin, Advanced Statictics (book)2nd edition (2015).